REMARKS

Applicant submits this Amendment in response to the Office Action mailed on August 9, 2007, which Office Action contained a final rejection of all claims, and following a telephonic interview with Examiners Ramachandran and Padmanabhan on October 9, 2007. Applicant submits herewith a Request for Continued Examination (RCE) along with the applicable fees.

The claims have been amended as follows. As discussed during the interview, claim 1 has been amended to call for the administered selective estrogen beta receptor agonist to have a relative potency for estrogen receptor beta compared to estrogen receptor alpha higher than that of genistein. Support for this amendment is found in the specification on page 5, last 5 lines, in Figure 3, and in Example 8.

Rejections of the Claims under 35 U.S.C. §103(a)

A. Claims 1-6, 8-12, 15 and 16 in view of Hermsmeyer, U.S. Patent No. 6,056,972; Meyers, J. Med. Chem., 44:4230-4251 (2001); and Weihua, PNAS, 99:13589-13594 (2002)

The Examiner has rejected claims 1-6, 8-12, 15 and 16 under 35 U.S.C. §103(a) as being obvious in view of the combined disclosures of Hermsmeyer, U.S. Patent No. 6,056,972; Meyers, J. Med. Chem., 44:4230-4251 (2001); and Weihua, PNAS, 99:13589-13594 (2002). Applicant traverses the rejection of these claims on this ground.

Hermsmeyer discloses that estradiol 17β reduces coronary artery reactivity and that estradiol 17β, in combination with progesterone, reduces coronary vasospasm in monkeys. Myers is cited for its disclosure that estradiol 17β is more potent towards estrogen beta receptors

than is genistein and that diarylpropionitrile is an estrogen beta receptor selective ligand. Weihua is cited for its disclosure that androstane is an estrogen beta receptor selective ligand.

The Examiner, pages 3-4, bridging paragraph, states that:

> It would have been obvious to one of ordinary skill in the art at the time of the invention to administer diarylpropionitrile (DPN) for estradiol 17β. The motivation to do so is provided by Meyers et al. Minshall teaches that vascular hyperreactivity and the ability to provoke coronary vasospasm can be normalized by adding physiological levels of estradiol and Meyers teach that the DPN has more potency towards ER β ligand. Hence by administering DPN, an estrogen receptor beta agonist that is more potent than estradiol a person of ordinary skill in the art would have been motivated by the expectation of success and in achieving at least similar or superior therapeutic benefits in the treatment of vascular hyperreactivity compared to estradiol.

Applicant respectfully disagrees and suggests that the above reasoning of the Examiner is impermissibly based upon hindsight following a reading of the present application.

As stated by the Examiner, the Hermsmeyer patent discloses the usefulness of estradiol 17β to reduce coronary artery hyperreactivity. Estrogen 17β, however, is not a selective estrogen beta receptor agonist, as called for in the present claims. Rather, estrogen 17β interacts with a variety of estrogen receptors.

The Meyers reference cited by the Examiner, in Table 4 on page 4241 and in Table 5 on page 4241, sets forth the relative activity of estradiol for estrogen alpha receptor and estrogen beta receptor. As disclosed in these two tables, estrogen is not a selective estrogen beta receptor agonist, as called for in the present claims. Meyers discloses that estradiol has a higher potency for estrogen alpha receptors than for estrogen beta receptors. The ratio of β:α for estradiol is 0.46. Thus, estradiol has a potency of about 2 to 1 in favor of estrogen alpha receptor and, therefore, even though estradiol has high potency on the beta receptor, estradiol would be

considered to be non-selective or could possibly even be considered to be a selective estrogen alpha receptor agonist.

The present invention lies in the discovery that, although estradiol has been known to have favorable vascular effects, the full agonist effect of estradiol is not necessary to obtain these benefits. Rather, it has been discovered that compounds that are selective estrogen beta receptor agonists effectively provide these same benefits.

Estradiol has an agonistic effect on multiple receptors. The cited Meyers reference teaches the presence of estrogen alpha and beta receptors. Submitted herewith is Haas et al, Hypertension, 49:1358-1363 (2007). Haas discloses another estrogen receptor, referred to as GPR30, that is present in arteries and veins. This receptor is a protein that is structurally unrelated to the estrogen alpha or estrogen beta receptor. Whereas the estrogen alpha and beta receptors are nuclear receptors, GPR30 is a membrane-associated receptor. Submitted herewith is Toran-Allerand, Endocrinology, 145(3):1069-1074 (2004). Toran-Allerand discloses another estrogen receptor, referred to as ER-X, that has been identified in various neural tissues and in lung. Like the GPR30 receptor, ER-X is a membrane-associated receptor.

It is submitted that, whereas the cited Hermsmeyer reference discloses that estradiol, which activates all estrogen receptors, reduces coronary artery hyperreactivity, it is the present application that discloses that this reduction in coronary artery hyperreactivity may be obtained by preferentially activating the estrogen beta receptor.

It is further submitted that this discovery is not suggested or disclosed in the prior art, when taken alone or in combination. Hermsmeyer makes no suggestion regarding any compound other than estradiol that activates estrogen receptors. Nor does Meyers and/or Weihua

suggest or disclose that selective activation of estrogen beta receptors is effective in reducing coronary artery hyperreactivity.

MPEP 2144.04(II)(B) states that, "Omission of an Element with retention of its function is an indicia of unobviousnes.," (Ex parte Wu, 10 USPQ 2031 (Bd. Pat. App. & Inter. 1989)). It is submitted that this statement precisely describes the present situation.

In the present case, it is known that estrogen administration provides a decrease in coronary vascular hyperreactivity. It is further known that estrogen binds to several receptors, including estrogen receptor alpha, estrogen receptor beta, estrogen receptor GPR30, and estrogen receptor ER-X. It is known also that estrogen has an activity in alpha receptors that is about twice that of beta receptors. See Meyers. In accordance with the present invention, it has been discovered that this binding to the alpha receptor is not necessary and that the result of decreasing coronary vascular hyperreactivity is obtained by use of a selective estrogen beta receptor agonist, such as those specifically recited in the claims.

In view of the fact that it is only the present application which teaches that an estrogen beta receptor selective compound is effective in reducing the incidence or severity of vascular hyperreactivity, it is submitted that the Examiner's finding that it is obvious to use a selective estrogen beta receptor agonist in place of a compound that has equal or higher selective potency for estrogen receptor alpha is improperly based on hindsight following a reading of the present application.

Applicant submits that the Hermsmeyer reference's disclosure of estrogen is not pertinent to the present application. Moreover, the secondary references do not fill in the gaps in

the teaching of Hermsmeyer. Therefore, it is submitted that the claims are patentably distinct over the combined teachings of Hermsmeyer, Meyer, and Weihua.

The above was discussed with the Examiners during the interview of October 10, 2007. It was agreed during the interview that an amendment of the claims to call for relative potency selectivity would overcome the rejection of the claims on this ground. Accordingly, in order to facilitate prosecution of the application, Applicant has amended independent claim 1 accordingly. In view of the above arguments and in view of the amendment of claim 1, the Examiner is respectfully requested to reconsider and to withdraw the rejection claims 1-6, 8-12, 15 and 16 on this ground.

B. Claim 7 in view of Hermsmeyer, U.S. Patent No. 6,056,972; Meyers, J. Med. Chem., 44:4230-4251 (2001); Weihua, PNAS, 99:13589-13594 (2002); and Barkheim, Molecular Pharmacology, 54:105-112 (1998)

The Examiner has rejected claim7 under 35 U.S.C. §103(a) as being obvious in view of the combined disclosures of Hermsmeyer, U.S. Patent No. 6,056,972; Meyers, J. Med. Chem., 44:4230-4251 (2001); Weihua, PNAS, 99:13589-13594 (2002); and Barkheim, Molecular Pharmacology, 54:105-112 (1998). Applicant traverses the rejection of these claims on this ground.

The cited Hermsmeyer, Meyer, and Weihua references are discussed above. Barkheim is cited for its disclosure that epiestrol is a selective estrogen beta receptor agonist. It is noted also that Barkheim, like Meyer, discloses that estradiol is not a selective beta receptor agonist. In fact, Barkheim discloses that estradiol has estrogen alpha selective agonist potency. See Abstract.

As discussed above, the combination of Hermsmeyer, Meyer, and Weihua does not disclose or suggest the present invention because the only teaching of a reduction in coronary arterial hyperreactivity in the cited references relates to estradiol and Meyer discloses that estradiol is not a selective estrogen beta receptor agonist, but rather is selective for the estrogen alpha receptor as much as, or even more than, for the estrogen beta receptor. Weihua relates to androstane.

It is submitted that combining the disclosure of Barkheim with the other cited references does not disclose or suggest the present invention. Barkheim, either alone or in combination with the other prior art, does not suggest that epiestriol has the function called for in the present claims.

Accordingly, Applicant respectfully requests the Examiner to reconsider and to withdraw the rejection of claim 7 on this ground.

C. Claims 13 and 14 in view of Hermsmeyer, U.S. Patent No. 6,056,972; Meyers, J. Med. Chem., 44:4230-4251 (2001); Weihua, PNAS, 99:13589-13594 (2002); and Burry, J. Obstet. Gynecol., 180:1504-1511 (1999)

The Examiner has rejected claims 13 and 14 under 35 U.S.C. §103(a) as being obvious in view of the combined disclosures of Hermsmeyer, U.S. Patent No. 6,056,972; Meyers, J. Med. Chem., 44:4230-4251 (2001); Weihua, PNAS, 99:13589-13594 (2002); and Burry, J. Obstet. Gynecol., 180:1504-1511 (1999). Applicant traverses the rejection of these claims on this ground.

The cited Hermsmeyer, Meyer, and Weihua references are discussed above. Burry is cited for its disclosure of transdermal application of estradiol.

As discussed above, the combination of Hermsmeyer, Meyer, and Weihua does not disclose or suggest the present invention because the only teaching of a reduction in coronary arterial hyperreactivity in the cited references relates to estradiol and Meyer discloses that estradiol is not a selective estrogen beta receptor agonist, but rather is selective for the estrogen alpha receptor as much as, or even more than, for the estrogen beta receptor. Weihua relates to androstane. Burry, like Hermsmeyer, pertains to estradiol.

It is submitted that combining the disclosure of Burry with the other cited references does not disclose or suggest the present invention. Burry, either alone or in combination with the other prior art, does not suggest that epiestriol has the function called for in the present claims.

Accordingly, Applicant respectfully requests the Examiner to reconsider and to withdraw the rejection of claim 13 and 14 on this ground.

CONCLUSION

Applicant submits that the claims, as amended herein, are in condition for allowance and request an early notice to that effect. A Request for Continued Examination is being submitted with this Amendment.

Respectfully submitted,

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Attachments: Haas et al, Hypertension, 49:1358-1363 (2007)
Toran-Allerand, Endocrinology, 145(3):1069-1074 (2004)

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450, on October 15, 2007.

Dated: 10/15/2007

Howard M. Eisenberg

Differential Effects of 17β-Estradiol on Function and Expression of Estrogen Receptor α, Estrogen Receptor β, and GPR30 in Arteries and Veins of Patients With Atherosclerosis

Elvira Haas, Matthias R. Meyer, Ulrich Schurr, Indranil Bhattacharya, Roberta Minotti, Hung H. Nguyen, Andres Heigl, Mario Lachat, Michele Genoni, Matthias Barton

Abstract—Venous complications have been implicated in the adverse effects of hormone replacement therapy. This study investigated acute effects of the natural estrogen, 17β-estradiol, on function, estrogen receptors/GPR30 expression, and kinase activation in vascular rings and cultured smooth muscle cells from arteries and veins of patients with coronary artery disease. Changes in vascular tone of internal mammary arteries and saphenous veins exposed to the steroid were recorded. 17β-Estradiol caused concentration-dependent, endothelium-independent relaxation in arteries ($P<0.05$ versus solvent control) but not in veins (P not significant). 17β-Estradiol enhanced contractions to endothelin-1 in veins but not in arteries. The novel membrane estrogen receptor GPR30 was detected in both vessels. Moreover, gene expression of estrogen receptor β was 10-fold higher than that of estrogen receptor α or GPR30 ($P<0.05$). Expression of all 3 of the receptors was reduced after exposure to 17β-estradiol in arteries but not in veins ($P<0.05$). Basal phosphorylation levels of extracellular signal-regulated kinase were higher in venous than in arterial smooth muscle cells and were increased by 17β-estradiol in arterial cells only. In summary, this is the first study to report that, in human arteries but not in veins, 17β-estradiol acutely affects vascular tone, estrogen receptor expression, including GPR30, and extracellular signal-regulated kinase phosphorylation. These data indicate that effects of natural estrogens in humans differ between arterial and venous vascular beds, which may contribute to the vascular risks associated with menopause or hormone therapy. (***Hypertension.* 2007;49:1358-1363.**)

Key Words: aromatase ■ bypass graft ■ clinical study ■ gender ■ hormone replacement therapy ■ human ■ 5α-reductase

Endogenous estrogens have been implicated in protection from cardiovascular disease in premenopausal woman, and accordingly lack of estrogens is thought to be in part responsible for accelerated development of atherosclerosis in men and postmenopausal women.[1] Although epidemiological studies have suggested a protective effect of postmenopausal hormone therapy on the arterial vasculature,[2] this concept has been challenged recently based on the results of randomized clinical trials using conjugated equine estrogens that were associated with increased venous complications.[1,3–5] Estrogens, including their physiologically most important form, 17β-estradiol, affect vascular homeostasis via nuclear estrogen receptors (ERs), ERα and ERβ, controlling cell growth, vascular tone, and thrombosis.[1,5–8]

In nonatherosclerotic human coronary arteries, 17β-estradiol induces rapid, endothelium-independent vasodilation[7] and enhances endothelium-dependent relaxation to bradykinin.[9] Vanhoutte and coworkers[10,11] reported that vascular reactivity of veins showing a high release of vasoconstrictor prostanoids differs from that of arteries. The same group also showed that chronic administration of sex steroids differently affects vasoreactivity in arterial and venous vascular beds of rabbits and pigs.[12,13] Acute effects of estrogens involve membrane-associated estrogen binding sites independent of nuclear activation of ERα and ERβ,[14,15] and it has been shown recently that ERα protein also localizes to the cell membrane.[16–18] In addition, a G protein–coupled, 7-transmembrane receptor termed "GPR30" was identified recently as a protein structurally unrelated to ERα or ERβ binding 17β-estradiol with high affinity.[19,20] Whether and at what level GPR30 is expressed in human blood vessels is not known. Also, there is no information about whether 17β-estradiol similarly affects ER expression, vasoreactivity, or intracellular signaling pathways in arteries and veins in

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humans, which would be important for the understanding of effects and adverse effects of estrogen therapy.

Therefore, in the present study, we investigated effects of 17β-estradiol, a nonselective agonist of ERα, ERβ, and GPR30,[21] in human mammary arteries and saphenous veins. We also investigated acute effects on gene expression of ERα, ERβ, GPR30, and enzymes involved in estrogen synthesis. Finally, basal phosphorylation levels and effects of 17β-estradiol on phosphorylation of the kinases extracellular signal-regulated kinase (ERK)1/2 and Akt were determined. The results indicate differences in ER expression and pronounced heterogeneity in the responsiveness to 17β-estradiol between arteries and veins. Unlike arteries, human veins display higher levels of basal ERK1/2 phosphorylation and were devoid of any changes in vascular tone, gene expression, or ERK1/2 phosphorylation on exposure to 17β-estradiol.

Methods

An Expanded Methods section is available in a data supplement available online at http://hyper.ahajournals.org.

Patients and Vascular Function Studies

Human internal mammary arteries (IMAs) and saphenous veins (SVs) were obtained from patients undergoing coronary artery bypass graft surgery. The study and the experiments were reviewed and approved by the institutional ethics committee, and informed consent was obtained from patients before surgery. The study conformed with the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. Clinical and laboratory data were collected from patient records, and low-density lipoprotein concentrations were calculated using the Friedewald formula.[22] Patient demographics, clinical parameters, and laboratory data values are shown in Table S1. Vascular function experiments were performed as described.[7] For experimental details see the data supplement.

Quantitative Real-Time PCR Gene Expression Studies

Selected rings were snap-frozen in liquid nitrogen after incubation with either 17β-estradiol or solvent control for 3 hours at 37°C and kept at −80°C until further analysis. For experimental details of RNA isolation, reverse transcription and real-time PCR, and primer sequences, see the data supplement.

Effects of 17β-Estradiol on Phosphorylation of ERK1/2 and Akt

Internal mammary artery and SV smooth muscle cells were explanted using the explant technique as described[23] and cultured in petri dishes using phenol-red free DMEM and Ham's F-12 medium (1:1, vol/vol; Bioconcept) supplemented with 10% FCS (Sigma Aldrich). Cells were identified by their hill and valley morphology using phase-contrast microscopy and immunofluorescence staining for α-actin.[23] Cells were passaged after treatment with 0.05% trypsin (weight/vol)/0.02% EDTA (weight/vol) in PBS. Subconfluent cells of passages 2 to 4 were used for experiments. Cells were serum-starved for 24 hours and then exposed to 17β-estradiol for 10 minutes. Western blot analysis experiments are described in the data supplement.

Calculations and Statistical Analyses

Data are expressed as means±SEM. Vasoconstrictor responses are given as percentage of contraction to KCl, and vasodilator responses were calculated as percentage of relaxation of precontraction as described.[7] For time-course experiments, curves are shown, but

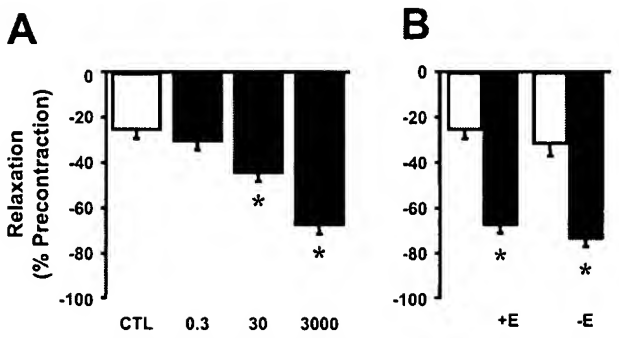

Figure 1. A, Relaxant effects to different concentrations of 17β-estradiol (0.3, 30, and 3000 nmol/L) compared with solvent control (CTL) after 40-minute exposure in human IMA. B, Denudation of arteries (-E) had no effect on maximal relaxant effect of 17β-estradiol after 40-minute exposure (−74+3% vs −70+3%; +E). Data are mean±SEM, *$P<0.05$ vs solvent control (CTL).

presented values (group means±SEM) were obtained at indicated time points after 17β-estradiol administration and were analyzed with unpaired Student's t test or, if data were not normally distributed, the Mann–Whitney U test was used. Concentration–response curves were analyzed by a 2-way ANOVA followed by posthoc unpaired multiple comparison test (Bonferroni test). Gene expression is expressed as arbitrary units ($\Delta\Delta C_T$ method).[24] Comparisons of group means were performed using the unpaired Student's t test or the Mann–Whitney U test if data were not normally distributed. Statistical significance was accepted at $P<0.05$.

Results

Direct Effects of 17β-Estradiol on Vascular Tone

In precontracted IMA rings, 17β-estradiol evoked a concentration-dependent relaxation starting at 30 nmol/L (−31±4%; $P<0.05$ versus solvent control; Figure 1A) reaching a maximal response of −70±3% at 3 μmol/L after 40 minutes ($P<0.05$ versus solvent control; Figure 1A). Similar dilating effects of 17β-estradiol were observed in endothelium-denuded IMA rings after 40 minutes (−74±3% at 3 μmol/L; $P<0.05$ versus solvent control; Figure 1B, "-E"). Onset of the relaxation was within 5 minutes after application of the hormone ($P<0.05$ versus solvent control; Figure 2A). In contrast, 17β-estradiol showed no relaxant effect in SV rings even after 40 minutes of exposure (Figure 2B).

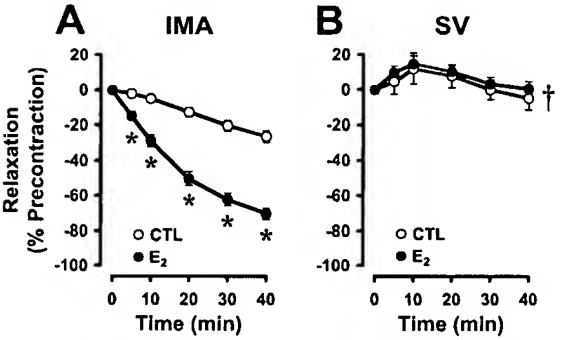

Figure 2. Time-dependent relaxant effects of 17β-estradiol in human IMA and SV at individual time points. Incubation with 17β-estradiol (E_2; 3 μmol/L) evoked time-dependent relaxations in precontracted IMA rings (A). In contrast, 17β-estradiol had no relaxant effect in SVs (B). Data are mean±SEM; n=18 to 22 per group for IMA; n=7 to 11 for group in SV; *$P<0.05$ vs solvent control (CTL), †$P<0.05$ vs IMA.

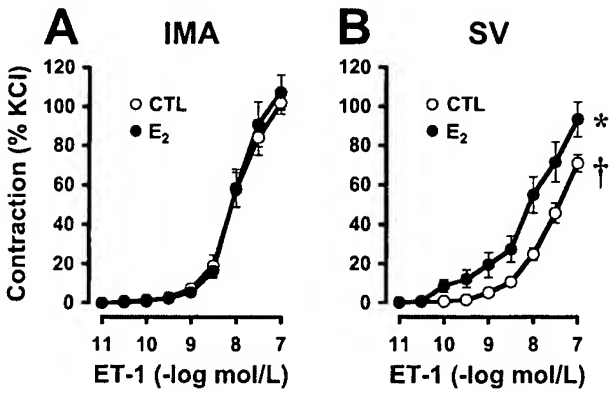

Figure 3. Effect of 17β-estradiol on endothelin-induced contractions in human IMAs and SVs. Preincubation for 30 minutes with 17β-estradiol (E_2; 3 μmol/L) increased contractions in SVs (B) but not in IMAs (A). Data are mean±SEM; n=9 to 14 per group; *P<0.05 vs solvent control (CTL); †P<0.05 vs IMA.

Effects of 17β-Estradiol on Contractile Responses

Contractions to endothelin-1 (0.01 nmol/L to 0.1 μmol/L) were stronger in IMA than SV rings (maximal response: 102±6% versus 71±4%; P<0.05). In SV rings, 17β-estradiol potentiated contractions to endothelin-1 (P<0.05 versus solvent control) but had no effect on contractions in IMA rings (Figure 3). Contractions to norepinephrine (0.1 to 3000 nmol/L) were unaffected by 17β-estradiol in IMA and SV rings (data not shown).

Effects of 17β-Estradiol on Endothelium-Dependent Relaxation

Endothelium-dependent responses to bradykinin (0.01 nmol/L to 10 μmol/L) were unaffected by pretreatment with 17β-estradiol (3 μmol/L) in IMA (42±5% versus 40±2% for maximal response; P not significant) and SV rings (54±6% versus 50±5% for maximal response; P not significant). Moreover, maximal relaxation and sensitivity of the endothelium-independent responses to sodium nitroprusside (0.01 nmol/L to 10 μmol/L) were similar in both IMA and SV and unaffected by incubation with 17β-estradiol (data not shown).

ER Gene Expression: Effects of 17β-Estradiol

In both IMA and SV, mRNA transcripts of ERα, ERβ, and GPR30 genes were detected. In IMA and SV, gene expression levels of ERβ were >10-fold higher than mRNA levels of ERα or GPR30 (Table). Moreover, expression levels of ERα and ERβ in IMA were 2.1-fold and 1.8-fold higher than in SV (P<0.05), whereas GPR30 was expressed at similar levels in both vessels. These differences in expression levels were also evident in rings not exposed to the solvent control (data not shown). Further, ERα, ERβ, and GPR30 genes were expressed in cultured smooth muscle cells derived from IMA or SV (data not shown). Exposure to 17β-estradiol reduced ERα, ERβ, and GPR30 gene expression in IMA (P<0.05 versus solvent control; Table), whereas 17β-estradiol had no effect in SV (P not significant; Table). In both IMA and SV, transcripts of aromatase and 5α-reductase type 1 were detected at comparable expression levels and unaffected by 17β-estradiol (P not significant; Table). 5α-Reductase type 2 mRNA was not detected in any of the samples investigated.

Effects of 17β-Estradiol on Phosphorylation of ERK1/2 and Akt

Phosphorylation of the kinases Akt (protein kinase B) and ERK1/2 was analyzed in IMA and SV smooth muscle cells after exposure to 17β-estradiol (10 to 1000 nmol/L) for 10 minutes by immunoblotting with phosphospecific antibodies. Basal phosphorylation of ERK1/2 was higher in unstimulated SVs than in IMA smooth muscle cells despite a similar level of total ERK1/2 (Figure 4). 17β-Estradiol enhanced ERK1/2 phosphorylation in IMA at low concentrations (10 nmol/L; Figure 5, left) but had no effect in SV smooth muscle cells (Figure 5, right). Akt phosphorylation was unaffected by 17β-estradiol in IMA and SV smooth muscle cells (Figure 5). In contrast, insulin caused strong phosphorylation of Akt (data not shown).

Discussion

This study presents several new findings contributing to the understanding of vascular action of estrogens in the human vasculature. The results demonstrate that SVs completely lack vasodilator effects, changes in ER gene expression, or kinase phosphorylation in response to 17β-estradiol; venoconstriction to endothelin-1 was increased. In contrast, in mammary arteries, short-term exposure to 17β-estradiol results in endothelium-independent relaxation, ERK1/2 phosphorylation, and downregulation of ERα, ERβ, and GPR30 gene expression. To the best of our knowledge, this study is also the first demonstrating that human blood vessels express the novel ER GPR30 and that arteries and veins differently

Gene Expression of ERs, GPR30, Aromatase, and 5α-Reductase Type 1

Vessel Treatment	ERα	ERβ	GPR30	Aromatase	5αRed1
IMA					
Solvent control	1.6±0.3	21.7±4.1*	1.5±0.2†	17.9±4.4	16.5±2.1
17β-Estradiol	0.9±0.1‡	11.2±1.3*‡	0.8±0.1†‡	14.5±3.7	13.8±2.2
SV					
Solvent control	0.8±0.1§	11.8±2.4*§	1.6±0.4†	16.9±2.2	11.6±2.3
17β-Estradiol	0.6±0.1	11.8±2.2*	1.3±0.3†	16.9±2.5	9.1±1.1§

5αRed1 indicates 5α-reductase type 1. Data are mean±SEM and are expressed as arbitrary units=$\Delta\Delta C_T$ of gene of interest and housekeeping gene GAPDH; n=7 to 12 per group.

*P<0.05 vs ERα; †P<0.05 vs ERβ; ‡P<0.05 vs control; and §P<0.05 vs IMA.

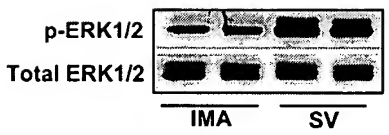

Figure 4. Basal levels of ERK1/2 phosphorylation in quiescent human IMA and SV smooth muscle cells.

respond to the natural estrogen 17β-estradiol at both the functional and molecular level.

Rapid dilator effects to 17β-estradiol, which is a nonselective agonist of ERα, ERβ, and GPR30,[21] involve nongenomic signaling and are thought to be mediated via membrane-bound estrogen binding sites.[15,25] Confirming previous studies in human coronary arteries,[7,26] the present study shows that 17β-estradiol causes endothelium-independent relaxation in human mammary arteries. The exact contributions of ERα and ERβ to the acute dilator response to 17β-estradiol in IMAs are currently unknown; however, recent work from our laboratory using epicardial coronary arteries suggests that the dilator response caused by selective activation of ERα is markedly different from nonselective ER activation.[27] In contrast to mammary arteries, we observed no dilation in response to 17β-estradiol in human SVs. This has potentially important implications, because hormone therapy has been associated with venous complications.[28] The mechanisms underlying this lack of responsiveness may be severalfold. Seminal work by Vanhoutte and coworkers[10,11] has shown that veins display different response patterns to various vasoactive substances compared with arteries and that veins show a higher release of vasoconstrictor prostanoids.[10] Recently, Eriksson et al[29] reported that proinflammatory activity is greater in veins than in arteries. Similar to our present findings, only weak dilator effects of 17β-estradiol have been observed previously in porcine veins in vitro.[13]

Based on our previous observation showing that 17β-estradiol acutely modulates the vascular activity of vasoconstrictors such as angiotensin or serotonin in human arteries,[9,30] we now compared the effects of 17β-estradiol on endothelin-mediated contractility between human arteries and veins. Endothelin is regarded as one of the most potent and long-lasting vasoconstrictors.[31] Although 17β-estradiol had no effect on contractions in internal mammary arteries, responses were enhanced in SVs, compatible with an indirect estrogen-mediated venoconstrictor effect. Endogenous sex hormones not only regulate endothelin expression[32] but also regulate venous endothelin receptor expression.[33] It is also of interest to note that venoconstriction in response to endothelin-1 involves the release of vasoconstrictor prostaglandins,[34] and 17β-estradiol may even stimulate the formation of cyclooxygenase-derived vasoconstrictor prostanoids.[12,35]

An important finding and to our knowledge the first demonstration that, in addition to the "classical" ERs, ERα and ERβ, was the observation that the novel membrane ER GPR30[19,20] is expressed in smooth muscle cells of human arteries and veins. Expression of ERβ was higher than that of ERα or GPR30; this is likely to be of relevance for vascular effects of estrogens and/or susceptibility to disease. It was been shown that expression of ERβ, but not of ERα, correlates with coronary artery calcification in women.[36] Surprisingly, in mammary arteries but not in veins, GPR30 mRNA, like ERα and ERβ mRNA, was downregulated after short-term exposure to 17β-estradiol. The mechanisms underlying this regulation are currently unclear. Interestingly, inactivation of transcription by methylation of ER genes differs between arteries and veins.[37] Also possibly relevant for adverse effects of estrogen is the observation that high ERβ expression in veins is associated with growth of vascular smooth muscle.[38]

In the present study, we demonstrate that human arteries and veins express aromatase and 5α-reductase type 1 but not 5α-reductase type 2. Given that testosterone is locally converted to 17β-estradiol by aromatase[39,40] and that aromatase deficiency accelerates atherogenesis in males,[41] it is possible that protective vascular effects of 17β-estradiol are not restricted to females. Indeed, androgens are converted to estrogens in males,[41] and estrogen activity is important for the atheroprotective effects of androgens in males.[42,43] This is further supported by the observation that the nonselective ER agonist 17β-estradiol inhibits experimental atherosclerosis in male mice.[41] Together with our previous findings in humans[7] and atherosclerotic mice,[44] the present investigation indicates that arteries from female and from male patients respond to 17β-estradiol via rapid changes in vascular tone, EKR1/2 phosphorylation, and ER expression.

Basal levels of ERK1/2 phosphorylation may vary between smooth muscle cells from different arterial vascular beds,[45] whereas no data on veins are available. We found that basal activation of ERK1/2, as measured by the phosphorylated protein, was higher in venous compared with arterial smooth muscle cells. We also found that 17β-estradiol induces ERK1/2 phosphorylation only in arterial but not in venous smooth muscle cells. Higher basal levels of phosphorylated ERK1/2 in veins may possibly explain the inability to further increase ERK1/2 phosphorylation in this vessel upon exposure to 17β-estradiol.

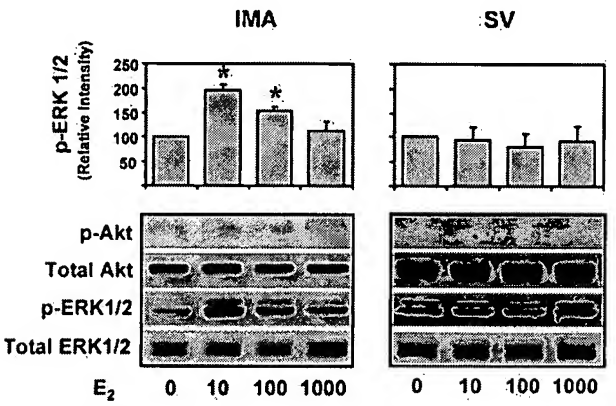

Figure 5. Effect of 17β-estradiol on phosphorylation of ERK1/2 and Akt in human IMA and SV smooth muscle cells in vitro. IMA (left) and SV (right) smooth muscle cells were exposed to 17β-estradiol (10 to 1000 nmol/L) for 10 minutes. Total Akt and ERK1/2 protein were used as a loading control. Densitometric evaluation of phosphorylated ERK1/2 from 3 independent experiments is shown. Data are mean±SEM; *$P<0.05$ vs solvent control.

Perspectives

We have demonstrated marked differences in functional and molecular responsiveness between human veins and arteries in response to the nonselective ER agonist 17β-estradiol. The results reported herein might add to the understanding of how natural estrogens or conjugated equine estrogens (which, among other substances, contain 17β-estradiol[3,4]) contribute to vascular protection and to vascular risk in humans.[46,47]

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Disclosures

None.

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Minireview: A Plethora of Estrogen Receptors in the Brain: Where Will It End?

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Until 1996, when estrogen receptor (ER)-β was discovered, life seemed simple. The gonadal steroid hormone 17β-estradiol had one receptor, the ER, a ligand-inducible nuclear transcription factor. ER variants, the result of base pair insertions, transitions, and deletions, as well as alternative splicing, were considered abnormal and a prominent feature of breast cancer. Since then, like many other scientific beliefs, this concept has increased dramatically in complexity, and we are now faced with an ever-increasing array of estrogen-binding proteins, putative ERs, in the brain as well as in the extraneural targets of estrogen. The end is unlikely to be in sight. Some of these putative receptors have been localized to plasma or nuclear membranes, and others to the cytoplasm and/or nucleus. The molecular characteristics of membrane ERs are still in question, and, in most instances, the proteins have not been sequenced or cloned. However, based on transfection and immunohistochemistry, the generally held view, if not dogma, maintains that both nuclear and plasma membrane-associated ERs probably originate from the same gene and transcript that produce the classical intranuclear receptors ER-α and ER-β. However, the physiological relatedness of this observation remains open to question. This review addresses evidence that, in addition to ER-α and ER-β, there exist a variety of non-ER-α/non-ER-β nuclear, cytoplasmic, and plasma membrane ERs in the brain, including G protein-coupled receptors; a novel, developmentally regulated, membrane-associated ER, ER-X; a functional, truncated ER-α variant, ER-46; and a putative ER that is immunochemically, structurally, and functionally completely distinct from ER-α and ER-β. (*Endocrinology* 145: 1069–1074, 2004)

Discovery consists not in seeking new landscapes but in having new eyes.

—Marcel Proust

BESIDES ITS WELL-ESTABLISHED organizational and activational actions on reproductive neuroendocrine function, estrogen also exerts a wide variety of actions on regions of the developing and adult brain that influence higher cognitive functions, pain mechanisms, fine motor skills, susceptibility to seizures, mood, temperature regulation, and sleep (1, 2). Despite the current journalistic hype surrounding the results of the Women's Health Initiative studies, clinical and experimental studies have shown that estrogen also has neuroprotective effects with respect to damage from Alzheimer's and Parkinson's diseases, multiple sclerosis, major depression and bipolar disorder, schizophrenia, and ischemic stroke (3–6). For at least three decades, this plethora of estrogenic actions in the brain was believed to be mediated by a single, ligand-activated transcription factor, the intranuclear estrogen receptor (ER) (7). The discovery in 1996 of a second form of the ER in rat prostate (8, 9), termed ER-β (the original ER is now referred to as ER-α), changed this view completely and opened a Pandora's box from which has emanated an increasing number of estrogen-binding proteins, putative ERs, often classified as alternative splicing variants; but some may even be new genes. A third, more distantly related member of the ER family, ER-γ, has also recently been cloned and is found only in teleosts (10).

The Classical Intranuclear ERs

Most of estrogen's known transcriptional actions in mammals are mediated by the classical receptor ER-α (7) and the more recently cloned ER-β (8, 9) whose role remains largely uncharacterized but may be modulatory. ER-α and ER-β are members of the nuclear receptor superfamily of ligand-inducible transcription factors whose family members include the steroid, thyroid hormone, retinoic acid, vitamin D, and nuclear orphan receptors (11–13). Under steady-state conditions, these receptors are predominantly intranuclear. ER-α and ER-β appear to be complementary but not redundant and are genetically and functionally distinct. It has been suggested that an important physiological role of ER-β is to modulate ER-α-mediated gene transcription by inhibiting ER-α-mediated gene transcription in the presence of ER-α, and partially replacing ER-α in its absence (14). Although ER-α and ER-β share DNA binding domains (97%), they differ somewhat with respect to their ligand-binding domains (LBDs) (60%) and bear virtually no homology within their N-terminal regions (9). ER-α and ER-β also differ to varying extents with respect to their binding affinities and ligand specificities and have distinct spatiotemporal patterns of expression (15). In the brain, for example, whereas neocortical ER-β is present throughout life (16), neocortical ER-α expression is developmentally regulated and normally expressed at very high levels only during the period of neocortical differentiation (17), suggesting a more restricted developmental role.

Abbreviations: AP-1, Activation protein-1; CLM, caveolar-like microdomain; ER, estrogen receptor; ERE, estrogen response element; ERKO, ER knockout; hsp90, heat shock protein 90; LBD, ligand-binding domain; pER, putative ER.

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ERs are kept in the inactive state by forming a complex with heat shock protein 90 (hsp90) (for reviews, see Refs. 12 and 13). In the traditional view of estrogen action, exposure of a target cell to estrogen initiates activation of its receptor and triggers a cascade of intracellular events, which includes phosphorylation on serine and tyrosine residues, dissociation of the ER from hsp90 with which the unbound receptor is complexed, and receptor dimerization (18). These multiple steps result in the direct interaction of the hormone-activated receptor dimers with a specific cognate regulatory DNA sequence in the promoter region of target genes [the estrogen response element (ERE)] or with other transcriptional factors (18–20) to regulate a wide variety of transcription factors, genes, and gene networks by either enhancing or suppressing their function.

Membrane-Associated ERs

Some estrogenic effects, however, cannot be attributed to ER-α or ER-β, which suggests the existence of additional subtypes. The traditional view of estrogen action explains inadequately the complete and extensive range of estrogen's actions in the brain, including the ability of estrogen to regulate many genes that do not exhibit an apparent ERE (21). In this regard, Kushner *et al.* (22) have shown that ERs not only bind to EREs in target genes to recruit a coactivator complex of cointegrator-associated protein-p160 that mediates stimulation of transcription but can also activate transcription at activator protein-1 sites that bind the Jun/Fos transcription factors via the activation protein-1 (AP-1) (23). Equally poorly explained are the mechanisms that underlie the very rapid effects of estrogen that occur within seconds to minutes (24–27). Such a rapid time course appears inconsistent with direct transcriptional modulation via classical intranuclear receptors, a process whose latency, although quite variable and dependent upon the size of the transcript and gene, nonetheless tends to be significantly longer than the seconds to minutes seen for the rapid effects of estrogen. For example, following aldosterone exposure, early genes were expressed 1 h after its addition (28). On the other hand, such rapid effects of estrogen could be explained by the presence of plasma membrane-associated ERs that may be coupled to downstream signal transduction pathways typically associated with rapid activation by growth factors, and in this way lead indirectly to the regulation of genes and transcription factors.

The existence of membrane-associated ERs has been highly controversial since 1977, when Pietras and Szego (29) described specific binding sites for estrogen at the outer surfaces of isolated endometrial cells. Controversy has persisted because of failures to isolate and characterize such a membrane-associated receptor protein(s). Nonetheless, strong functional evidence now exists for the presence and importance of plasma membrane ERs in a wide variety of neural and extraneural target cells of estrogen. Although ER-α and ER-β are thought to be largely intranuclear, plasma membrane-associated ER-α and ER-β have also been described (30–32). The prevailing view, if not dogma, maintains that both nuclear and plasma membrane-associated ERs probably originate from the same gene and transcript that produce ER-α and ER-β (30, 33). However, because this view is based largely on transfections of ER-α or ER-β into cells [CHO-K1 (30) and Rat2 fibroblasts (31)] that do not normally express these receptors, the extent to which such findings represent the physiological condition in cells that normally do express ER-α or ER-β is unknown. All the more so because we have recently shown that transfection of ERs into CHO-K1, COS-7, and Rat2 fibroblast cell lines is not necessary for rapid estradiol activation of the MAPK cascade (34). Contrary to the generally held opinion, these cell lines are not unresponsive to estradiol in their native, nontransfected state. Moreover, their estrogen responsiveness is associated with high-affinity estrogen binding (K_d, 1.8 nM), and with a wide variety of variously sized, specific protein bands on Western blots, which are immunoreactive with antibodies to ER-α and ER-β. These bands range in molecular mass from 32–76 kDa (CHO) and 32–109 kDa (Rat2), but do not include bands of 66/67 kDa (ER-α), or 55–60 and 64 kDa (ER-β) (34). Although the nature of these ER-α-like immunoreactive bands is unknown, they appear to be specific, because they can fully blocked by preadsorbtion with the immunizing peptide. Their association with the plasma membrane suggests that that they may represent novel, membrane-associated, estrogen binding sites.

Caveolae and Caveolar-Like Microdomains (CLMs) of the Plasma Membrane

In neurons, plasma membrane receptors have been reported to localize mainly to discrete CLMs (35). CLMs are the neuron-specific homologs of caveolae (36–38), which are microdomains associated with the plasma-membrane of most cell types other than neurons. Unlike caveolae proper, CLMs express the integral membrane protein flotillin (39) abundantly rather than the caveolar protein caveolin, whose expression in the brain is restricted to astrocytes and microglia (40). CLMs, like caveolae, are highly enriched in cholesterol, glycosphingolipids, sphingomyelin, and lipid-anchored membrane proteins, and have been implicated in signal transduction and lipid/protein trafficking. Some of the proteins reportedly concentrated within these aptly named "crowded little caves" (36), for example, include, among many others: 1) the classical ERs ER-α and ER-β, and the ER-α variant ER-46 (41–43), 2) receptor tyrosine kinases (*e.g.* the neurotrophin, insulin, epidermal growth factor and platelet-derived growth factor receptors), 3) the low-affinity neurotrophin receptor $p75^{NTR}$, 4) hsp90, 5) the src family of tyrosine kinases, 6) the docking/adaptor proteins Shc and Grb2, 7) signal transduction molecules such as members of the MAPK cascade [Ras, B-Raf (Rap1), MAPK kinase, and ERK], adenyl cyclase, protein kinase A, and protein kinase C, 8) G proteins and G protein-coupled receptors, 9) lipid signaling molecules, 10) endothelial nitric oxide synthase, 11) the amyloid precursor protein (44), and 12) glycosylphosphatidylinositol-anchored proteins. This pattern suggests that CLMs and caveolae may serve as functional signaling modules to compartmentalize, modulate, and integrate signaling events at the cell surface (37, 38).

Novel Membrane ERs

Although there is some evidence that transfected ER-α and ER-β may also behave as plasma membrane receptors (30, 31, 45), other studies document the involvement of novel plasma membrane ERs that are 1) neither ER-α nor ER-β (46–48), 2) G protein-coupled receptors (49–52), as well as 3) even an entirely different gene product with no relation to classical nuclear ERs that is structurally unique and exhibits intrinsic, ligand-stimulated, tyrosine kinase activity, as do growth factor receptors (53).

Reports of novel ERs are not new, although their identity has been based primarily on functional responses to estradiol, such as modulation of Ca^{2+} flux and K^+ channel activation (27) and activation of a variety of signal transduction pathways. Das *et al.* (47) showed that the effect of the catecholestrogen 4-hydroxyestradiol on uterine lactoferrin expression was not only mediated by a potentially novel ER but that ICI 182,780 inhibited this effect in wild-type, but not in ER-α gene-disrupted [ER knockout (ERKO)] tissue. Insensitivity to ICI 182,780 as well as to inhibitors of transcription and translation appears to be a feature of many rapid effects of estradiol on membrane receptors of both neural and extraneural targets that are not related to classical ER-α and ER-β (46–48). Other studies also support the existence of novel, ICI-insensitive ERs in the rapid and so-called nongenomic actions of estradiol in the brain (46, 47, 54). For example, 17β-estradiol-induced potentiation of kainate-induced currents was not blocked by ICI 182,780 in isolated hippocampal CA1 neurons of both wild-type and αERKO mice (46, 54). Similarly, high-affinity estrogen binding sites in pancreatic β-cells (48) and 17α- and 17β-estradiol activation of the MAPK family members ERK1 and ERK2 in neocortical explants were not blocked by the ICI compound (55). Although one may question whether the inability to block with the ICI compound is more likely the result of a nonspecific membrane effect than a characteristic of certain novel plasma membrane receptors, it should be pointed out that the ICI-insensitive receptors described above appear to be novel, high-affinity estrogen binding sites. Moreover, blocking by ICI 182,780 may not even be a universal response of the classical ERs. Thus, whereas ICI 182,780 decreased the expression of ER-α in rat testis and its efferent ductules, it was without effect on testicular ER-β (56). There is even a report of regional variations in antagonism by ICI 182,780 (57).

On the other hand, it has been reported that estrogen activation of cAMP response element-binding protein (58) and estrogen-mediated neuroprotection against β-amyloid toxicity (59) were completely blocked by ICI 182,780. Although this may well suggest an ER-α- or ER-β-dependent mechanism, it should be pointed out that, in both studies, the cell lines used were stably transfected with ER-α or ER-β, which, not surprisingly, would be blocked specifically by the ICI compound.

ER-X

To add to this increasing ER complexity, we have recently identified a novel and unique, plasma-membrane-associated putative ER that is neither ER-α nor ER-β. I have designated this ER, ER-X (60). ER-X is developmentally regulated and highly enriched in purified CLMs of postnatal d-7, but not adult, neocortical plasma membranes not only of wild-type, but also of αERKO (56) and, most importantly, of ER-α-null (61) mice (Nethrapalli, I., and D. Toran-Allerand, unpublished observations).

We have also recently identified ER-X in the neocortex, hypothalamus, cerebellum, and lung of the term fetal baboon (Nethrapalli, I., and D. Toran-Allerand, unpublished observations). The apparent molecular mass of ER-X (~62–63 kDa) in the rat, mouse, and baboon differs from that of ER-α (67 kDa) and ER-β (54–60 and 64 kDa). In developing neocortex, ER-X sometimes appears as a 62- to 64-kDa doublet. The 62-kDa portion is developmentally regulated, whereas the 64-kDa band may be found in the adult (Nethrapalli, I., and D. Toran-Allerand, unpublished observations). Mass spectroscopy, which is currently in progress, will definitively establish the molecular mass of ER-X.

ER-X binds [^{3}H]estradiol with high affinity but with binding properties and ligand specificities quite distinct from ER-α: its K_d of 1.6 nM is approximately one order of magnitude less than that of ER-α and ER-β. Although 17α-estradiol and 17β-estradiol compete equally well for binding; progesterone competes (50%) for membrane estradiol binding. This differs completely from the inability of progesterone to displace estradiol from ER-α.

Notwithstanding its immunoreactivity with antibodies to the C-terminal region of ER-α or the fact that an oligonucleotide probe to that same portion of the C-terminal region of ER-α hybridizes to ERKO neocortical neurons, ER-X is clearly not ER-α (60). ER-X exhibits some but not complete homology with the ER-α LBD, but has no homology with the N-terminal region. Thus, although the ER-α and ER-X proteins can be identified with the same antibodies to the ER-α LBD (MC20 antibody; Santa Cruz Biotechnology, Santa Cruz, CA), the immunoreactive ER-α band on a Western blot, for example, can be blocked completely by a 200- to 500-fold excess of the blocking MC20 peptide, whereas the immunoreactive MC20 ER-X band requires a 2000-fold excess (10 times more) of the peptide to be blocked fully.

ER-X is the receptor that mediates 17α-estradiol and 17β-estradiol activation of MAPK/ERK in developing neocortical explants, whereas ER-α- and ER-β-selective ligands do not elicit activation of MAPK/ERK and are either inhibitory (ER-α) or without effect (ER-β) (60). Although both 17α-estradiol and 17β-estradiol bind ER-X, 17α-estradiol appears to be the endogenous ligand of ER-X and activates MAPK/ERK at 1 pM. Significantly higher levels of 17β-estradiol are required for ERK activation in wild-type neocortex, perhaps reflecting the need to overcome, in addition, the inhibitory effect of ER-α, which, unlike 17α-estradiol, 17β-estradiol activates as well (60). As found with other constitutive membrane ERs, rapid activation of MAPK/ERK is not blocked by inhibitors of transcription or translation or the selective ER-α/ER-β antagonist ICI 182,780. Moreover, many characteristics of ER-X are the complete opposite of those attributed to ER-α and ER-β. For example, association of ER-X with hsp90 is an absolute requirement for estradiol activation of MAPK/ERK (62), whereas, in contrast, association with hsp90 is required to keep ER-α in the inactive state (12, 13, 63).

Preliminary studies (Sétáló, Jr., G., and D. Toran-Allerand, unpublished observations) suggest that ER-X has features of a G protein-coupled receptor. Pretreatment of neocortical explants with low doses of pertussis toxin (1 ng/ml, for 60 min), but not cholera toxin (1 μg/ml, for 60 min), completely abrogated the ability of 17α- and 17β-estradiol to elicit ERK1/2 phosphorylation. This pattern is consistent with possible involvement of $G_{\beta\gamma}$ subunits of the $G_{i/o}$ family and is also supported by preliminary results that suggest that 17α-estradiol and 17β-estradiol increase guanosine 5′-*O*-3-thio-triphosphate membrane binding, a prominent feature of G protein-coupled receptor activation by agonists. ER-X is up-regulated in adult mouse models of Alzheimer's disease and Down's syndrome (our unpublished observations), in adult ischemic brain injury (60) and in the pregnant uterus, from which it disappears shortly after parturition (Nethrapalli, I., and D. Toran-Allerand, unpublished observations). Based on analyses using 5′ rapid amplification of cDNA ends and RT-PCR (Tinnikov, A., and D. Toran-Allerand, unpublished observations), the evidence thus far suggests that ER-X is not an alternative splicing variant of ER-α or ER-β and may be a new gene. However, definitive proof awaits cloning the gene and sequencing the protein, which are both currently in progress.

Still More ERs

Other putative estrogen-binding proteins have also been identified in the brain. These include the identification of 112- and 116-kDa ERs in the adult rat cerebral cortex whose levels change with age and hormonal treatment but whose function is unknown (64). Ramirez and colleagues (65–67) have identified three membrane estrogen-binding proteins: 1) a 37-kDa protein with 100% homology with glyceraldehyde-3-phosphate dehydrogenase (65, 66), 2) a 55-kDa protein identified as β-tubulin whose binding was completely displaced by 17β-estradiol at 10^{-7} M (65), and 3) a 23-kDa protein identified as the oligomycin-sensitivity conferring protein (67). Their roles in estrogen-mediated actions are similarly unknown.

In addition, a 46-kDa amino-terminal truncated product of full-length ER-α, ER46, has been identified in the plasma membrane, cytosol, and nucleus of resting, estrogen-deprived, nonneural cells (43), but does not seem to have been sought for in the brain. ER46 modulates membrane-initiated estrogen actions, including endothelial nitric oxide synthase activation in endothelial cells, which it reportedly does more efficiently than full-length ER-α (43).

Complicating matters is the recent identification in the brain and other tissues of a heterodimeric estrogen-binding protein, termed the putative ER (pER) (81–84 kDa) (68). pER consists of two covalently bound subunits (61–67 and 17–27 kDa) and has been localized on the plasma or nuclear membrane of some cells, and in the cytoplasm and/or nucleus of others. pER has a high affinity for 17β-estradiol (K_d, 0.7 nmol) but does not bind other natural steroids, synthetic estrogens, or antiestrogens. A serine phosphatase, this receptor is immunochemically, structurally, and functionally completely distinct from ER-α, ER-β, or ER-γ. Immunoreactive pER is undetectable in reproductive organs (except the ovary), but has been localized in brain, muscle, blood vessels, and retina, as well as in mammary, endometrial, and prostate tumors. Anti-pER antibodies do not recognize ER-α or ER-β, whereas antibodies to ER-α or ER-β do not react with pER. Immunosuppressants, neuroleptics, and carcinogens influence [^{3}H]estradiol binding to pER. The anti-pER antibody reacts with calcineurin, a brain phosphatase, and anticalcineurin antibodies react with pER. It has been suggested that pER may mediate estrogenic actions in nonreproductive organs.

Light-Microscopic and Ultrastructural Localization of ERs

Specific binding of estrogen to the plasma membrane in brain was first shown in the 1980s by [^{3}H]estradiol binding to synaptic membranes (69). Since then, numerous studies, particularly in the hippocampus and hypothalamus, have documented plasma membrane and cytoplasmic localization of ER-α immunoreactivity at both light-microscopic (70–72) and electron-microscopic levels (73–75). ER-α-labeled profiles have been described as unmyelinated axons, axon terminals containing numerous small, synaptic vesicles, dendritic spines, and astroglial processes. Within dendritic spines, most ER-α immunoreactivity has been seen in plasmalemmal and cytoplasmic regions of the spine heads and interpreted as plasma membrane ER-α (73–75). However, the discovery of ER-X, which, like ER-α, has also been localized to the plasma membrane of dendritic spines with many of the same antibodies (Fig. 1; Ref. 60), makes this interpretation

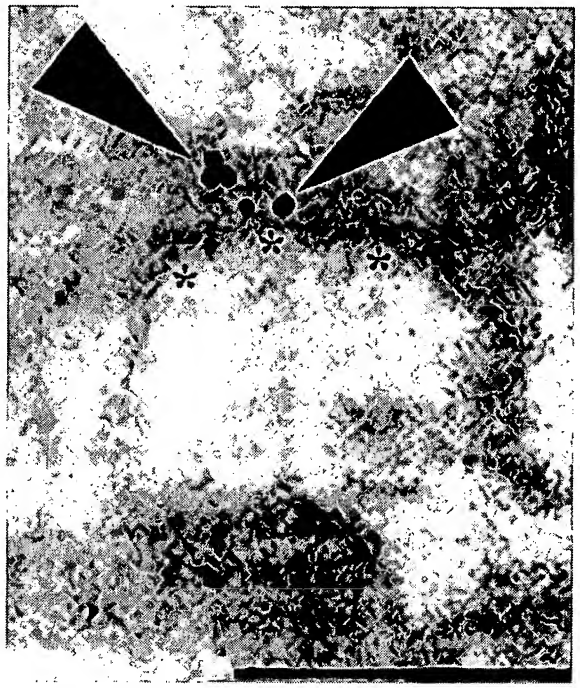

FIG. 1. Association of the 62- to 63-kDa ER-X protein with CLMs of the plasma membrane. Electron-microscopic double immunolabeling of an ultrathin cryostat section of postnatal d-7 ERKO neocortex shows the colocalization of ER-α-like immunoreactivity [dark reaction product (horseradish peroxidase); ***] with flotillin immunoreactivity (immunogold beads; *arrowheads*) on a mushroom-shaped neocortical dendritic spine. *Scale bar*, 10 μm. [Modified from C. D. Toran-Allerand, X. Guan, N. J. MacLusky, T. L. Horvath, S. Diano, M. Singh, E. S. Connolly, Jr., I. S. Nethrapalli, and A. Tinnikov. J Neurosci 22:8391, 2002 (60).]

open to question, particularly in the developing brain. The unfortunate but inevitable reliance on immunoreactivity to identify ER phenotypes has increased this confusion, because the antibodies to ER-α most frequently used are directed against the LBD of ER-α and recognize not only ER-α but ER-X as well as the ER-α-like immunoreactive bands in CHO, COS, and Rat2 fibroblasts.

A Plethora of ERs in the Brain: Where Will It End?

The nature of the receptor(s) involved in rapid estrogen actions remains elusive, and trying to unravel the receptors mediating these responses in the brain has proved daunting. This problem is compounded by the possibility that there may be a variety of additional membrane estrogen binding sites in the brain unrelated to ER-α and ER-β or to those described above, similar to the catecholaminergic receptor of pancreatic β-cells (48) and the 29-kDa membrane ER of sperm (76). If the membrane ERs of these extraneural estrogen targets are any indication, there may well be additional membrane ERs in the brain whose identity may vary with brain region, cellular phenotype, and developmental stage. The identification of a plethora of putative ERs in the brain suggests that one should keep a very open mind and radically revise the current view of estrogen actions in developing and adult estrogen target tissues, both with respect to the estrogens that elicit them and the receptors, other than ER-α- and ER-β, that may mediate them.

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